The Cytoprotective Effect of Crocin on Oxaliplatin Treated Colorectal Adenocarcinoma cell line in Vitro Study

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Abstract: Colorectal tumor is the third most regular malignancy and the third driving reason for growth demise in men and woman in the United States. In 2014, an expected 71,830 men and 65,000 women will be determined to have colorectal tumor and 26,270 men and 24,040 women were died from this disease. More prominent than 33% of all passing's (29% in men and 43% in woman). There is considerable variety in tumor area by age. For instance, 26% of colorectal growths in ladies matured more youthful than 50 years happen in the proximal colon, contrasted and 56% of cases in ladies matured 80 years and more seasoned.

Introduction:

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There are many types of treatment to the colorectal cancer (CRC) like surgery, radiation, chemotherapy, immunotherapy and others. Each of these above treatments has its indication effect and has Sid effect and / or adverse effect. The chemotherapy is one type of this treatment to the CRC that have the indication effect, side effect and adverse effect. There are many type of chemotherapeutic agent that used for this type of cancer one of them it is platinum compound (cisplatin, oxaliplatin and others).

There are two types of limitation in the use of these agents one of them is the side effect which is neurotoxicity, Ototoxicity, nephrotoxicity, and etc. and the second one is the resistance of cancer cells to these agents.

Keywords: Crocin, Colorectal adenocarcinoma, Oxaliplatin, Vitro study.

Aim of the study

Taste the cytoprotection effect of Crocin on oxaliplatin treated colorectal adenocarcinoma.

Material and methods:

By using the tissue culture techniques which is a newly used techniques to check the cytotoxicity of many compound and the expected mechanism of action these techniques made a good jump in the research in many field

In this study we use SW-480 cell line and after exposure of this cell line firstly to different concentrations of oxaliplatin (1, 0.5, 0.25, 0.125, 0.062, 0.031 mg/ml) in different times for 24hr,48hr and 72hr , secondly to different concentrations of Crocin (6, 3, 1, 0.5, 0.25, 0.125, 0.062, 0.031 mg/ ml) in different times for 24hr and 48hr. And thirdly to mix concentrations of oxaliplatin and Crocin in 48hr for the SW480 and and measure the cytotoxicity of these exposures by crystal violet test (C.V. test) to show and compare the effect of these agents specially the Crocin.

The oxaliplatin has cytotoxic effect, its inhibitory concentration 50% (IC50) in 24hr. is (0.125mg/ml) and in 48hr. the IC50 is (0.031mg/ml) which is the maximum cytotoxic effect in vitro. While the Crocin don't have cytotoxic effect at this tested concentration and for this cell line. After mixed different concentration of Crocin with different concentration of oxaliplatin the cytotoxicity of oxaliplatin reduce significantly.

Crocin

Chemical Name Crocin; Alpha-Crocin; Gardenia Yellow; Crocin 1; Crocin; Crocetin digentiobiose ester

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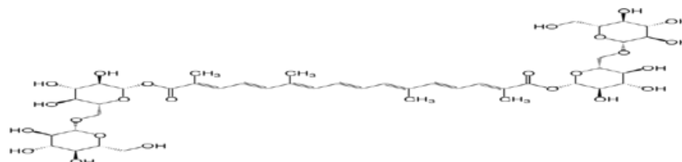
Molecular formula; $C_{44}H_{64}O_{24}$

Molecular weight; 976.96456 g/mol



1. Crocin occurrences in plants

Genus species	family	Common name	part
Crocus sativus	Iridaceae	saffron	plant
Cardenia jasminoides	Rubiaceae	jasmin	fruit

Therapeutic Uses

Cytoprotective, anti inflammatory, free radical scavenger activity

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Crocin improve spatial cognitive abilities following chronic cerebral hypo perfusion and that these effects may be related to the antioxidant effects of these compounds (Hosseinzadeh et al., 2012)

the action of saffron extract (crocin.) as anti oxidant on oxidative stress following renal ischemia-reperfusion injury (IRI) that lead to the generation of reactive oxygen species and lipid per oxidation are associated with tissue injury after the ischemic reperfusion ,therefore, the use of antioxidants appears rational in the improvement of kidney diseases therapy. (Hosseinzadeh et al., 2005)

Many of these pharmacological action of saffron is due to its crude component like crocetin, Crocin and others that have potent antioxidant and free radical scavenger activity against a many of radical oxygen species and pro-inflammatory cytokines. (Mashmoul et al., 2013)

Anti tumor, anti proliferative action

Crocin is mainly extracted from Crocus sativus L., it is have anti tumor, antiproliferative effects on cancer cells, but the mechanisms of action are poorly understood it is showed that telomerase activity of HepG2 cells decreases after exposures with crocin, which is may caused by decreased the expression of the catalytic subunit of the enzyme.(Noureini and Wink, 2012) the results indicated that the crocin interact with DNA and induce some conformational changes in the duplex.(Bathaie et al., 2007)

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The crocin have a wide range of anti cancer action against a wide range of cancer murine tumors and human leukemia cell lines. The present report surveys the crocin serving as a chemo preventive agent. Dosage dependant cytotoxic effect of crocin to carcinoma, sarcoma and leukemia cells in vitro was noted. The crocin inhibite ascites tumor development and expanded the life expectancy of the treated mice contrasted with untreated controls by 45-120%. What's more, it decreased the onset of papilloma development, diminished rate of squamous cell carcinoma and delicate tissue sarcoma in treated mice. It shows up now that saffron (dimethyl-crocetin) disturbs DNA-protein cooperation's e.g. topoisomerases II, essential for cell DNA amalgamation

Anticancer activity of crocin against a wide spectrum of murine tumors and human leukemia cell lines. The present report reviews the role of saffron in serving as a chemo preventive agent in modifying cancer risk. Dose-dependent cytotoxic effect to carcinoma, sarcoma and leukemia cells in vitro was noted. Saffron delayed ascites tumor growth and increased the life span of the treated mice compared to untreated controls by 45-120%. In addition, it delayed the onset of papilloma growth, decreased incidence of squamous cell carcinoma and soft tissue sarcoma in treated mice. Understanding the mechanisms of action of saffron has been solitarily based on their carotenoid-like action. Our results indicated significant inhibition in the synthesis of nucleic acids but not protein synthesis. It appears now that saffron (dimethyl-crocetin) disrupts DNA-protein interactions e.g. topoisomerases II, important for cellular DNA synthesis.(Nair et al., 1995)

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Crocin is the major constituent of Crocus sativus its highly inhibited the colorectal cancer and not affecting on the normal tissue. Crocus sativus extract mainly the crocin should be investigated for the treatment of colorectal cancer on three colorectal cancer cell lines (HCT-116, SW-480, and HT-29).(Aung et al., 2007)

2. Methods :

2.1 Cell lines used: we can know the types of the cell lines that used in this study and its properties

A. SW-480:

Organism Homo sapiens, human

Tissue colon

Morphology epithelial

Culture Properties adherent

Disease Dukes' type B, colorectal adenocarcinoma

Complete Growth Medium RPMI 1640

Cell Line Description Derived from a grade 3-4 colon adenocarcinoma. The initial cultures contained a mixture of epithelial and bipolar cells, but subsequently epithelial cells predominated**. (ATCC)**

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3. Preparation of reagents and solution (Kenkel, 2010) (Freshney and Freshney, 2004)

a. Phosphate buffered saline (PBS).

The substance of one vial of PBS was disintegrated in 1L. Of deionised distilled water (D.D.W) and blended with mixing. Disinfection was finished via autoclaving at 120 C° for 20 minutes and store in sterile beaker until use

b. Antibiotics stock solution

Garamycin stock solution

Garamycin vial of 80 mg/ 2ml solution was considered as stock solution stored at 4C° for uses.

d. Sodium hydroxide (1N) solution

1N of NaOH was set up by expansion of 40 grams of NaOH to roughly of 900 ml deionised water(DDW) with cooling and mixing until it broke down totally, then it was finished to 1L. Disinfection was finished by filtration through 0.2 µm Nalgene filter.

Hydrochloric acid (1N) solution

1N of HCL was set up by expansion of 58.8 ml of the 36% HCl drop by drop to 900 ml of deionised water (DDW) in volumetric beaker with persistent blending. At that point it was finished by deionised water into 1L. Disinfection done by 0.22 µm Millipore filter unit, and kept sterile until need

f. Trypsin-EDTA solution

A weight of 12.4 gm of Trypsin-EDTA powder (0.05% w/v Trypsin + 0.53mM EDTA) was dissolved in 900ml of D.D.W., with continuous mixed via stirring. And then the acidity of the solution (pH) adjusted to 7.2. And then the volume of solution completed to 1 L by DDW. The solution was sterilized by filtration using 0.45 and 0.22 µm Nalgene filters. The solution stored at - 20C° and dissolve to 37 C° when used.

g. Trypan blue dye

A stock solution of trypan blue dye (4mg/ml) was prepared by mixing PBS to 0.4 g of trypan blue into a gradated chamber with blending until it broke down. The volume was finished with PBS to 100 ml and put away at room temperature.

4. Preparation of the media

4.1. Tissue culture media

Liquid medium was prepared from powdered medium according to the US biological product manual as following:

A 16.650g of RPMI powder with HEPES support, L-glutamine and phenol red was broken down in roughly 900 ml of deionised water in volumetric beaker. Alternate segments include: 2g sodium bicarbonate powder or as indicated by need, 2.5 mg amphotercin B dry powder, 1.25 ml gentamycin arrangement and 0.5ml streptomycin arrangement had been included with constant mixing. The volume approximated to around one litter and the pH of the media changed in accordance with 7.4 by utilizing 1N NaOH and a pH meter. The volume was finished to one litter by including D.D.W. sterilization was finished by 0.4 and 0.2 µm Nalgin filter in this manner.

5 ml of the media was hatched in sterile flask for 4 days with day by day examination for indications of bacterial and contagious tainting. It was viewed as clean just if there should arise an occurrence of no indications of tainting amid four days of incubation. At that point the media was put away at 4ºC in until utilize

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4.2. Maintenance media

Its preparation was similar to those of tissue culture media listed above except it was free of fatal bovine serum (Freshney and Freshney, 2004)

4.3. Freezing media

10 ml stock solution was prepared from different constituents including: 6 ml serum free media, 3 ml fatal, bovine serum, 1ml dimethyl sulfoxide (DMSO) was added drop by drop with mixing. The stock was stored at - 20C° in between uses. (Riddle et al., 1993)

7. Crystal violet assay

Crystal violet (CV) assay was utilized to decide the optical thickness of the cell growth in every well of the micro titer plate, by utilizing plate spectrometer. After the end purpose of cytotoxicity examine, the maintenance medium with the test material was disposed of out and the wells washed with 100 µl of icy PBS via programmed pipette. At that point the cell cultures were fixed with 10 % buffered formalin for 20 min at room temperature. Fixative arrangement was disposed of and 100 µl of 0.1 % CV solution was added to every well. The specimens were brooded at room temperature for 20 min with shaking gently . After that the plates were washed by submersion in streaming tab water for 15 min. Micro plates were permitted to air dry and 0.2 % Triton X-100 in water was added to every well and brooded for 30 min at room temperature with delicate shaking to break up the color. At that point, 100 ml from every well was moved into another 96-well micro plate and the absorbance was examined at 570nm by a micro plate spectrometer (Castro-Garza *et al.*, 2007)

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The percentage of viability was calculated according to the following equation:

Viability% = optical density of test well/ optical density of control X 100.

The percentage of viability was calculated according to the following equation: *(Chiang et al., 2003)*

Inhibition % = (optical density of test wells/optical density of

Control wells) x 100

8. Cytotoxicity assays

As indicated by Freshney (2004), the cytotoxicity measures were connected for examined the effect of oxaliplatin and Crocin on SW-480 cell line culture. Different groupings of oxaliplatin and Crocin were tried for a specific time terms. At the point when the growth in the flask got to be as monolayer before it achieved the exponential stage, the cell monolayer were reaped and re-suspended with a growth medium in a centralization of $5X\ 10^5$ cell/ml and seeded in a 96 well microtiter plate.

Experiment

Measuring the cytotoxic effect of different concentration Of oxaliplatin on the cell line after 24, 48 and 72 hr and calculate the LD50 of oxaliplatin in sw-480 and Vero cell line. Measuring the cytotoxic effect of different concentrations Of Crocin on the cell line after 24, 48 and 72 hr and calculate the LD50 of Crocin if it found in sw-480 and Vero cell line. Measuring the cytotoxic effect of mixing different concentrations of Crocin with different concentration Of oxaliplatin on

the cell line after 24 and 48 hr and calculate the net effect for them in sw-480 and Vero cell line.

Results:

3.1. in vitro cytotoxicity study:

3.1.1. Effects of oxaliplatin cytotoxicity on SW-480 cell line (crystal violet assay) (C.V.):

Data in table (3-1) revealed that the viability percent of SW-480 cell line in the presence of different concentrations of oxaliplatin (1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.0078 mg/ml) was ranged between (36.07±4.51 -90.81±3.06) after 24h incubation,(21.16±0.84 - 59.82±2.52) after 48h incubation and (20.78±0.48 - 59.36±0.83) after 72h incubation.

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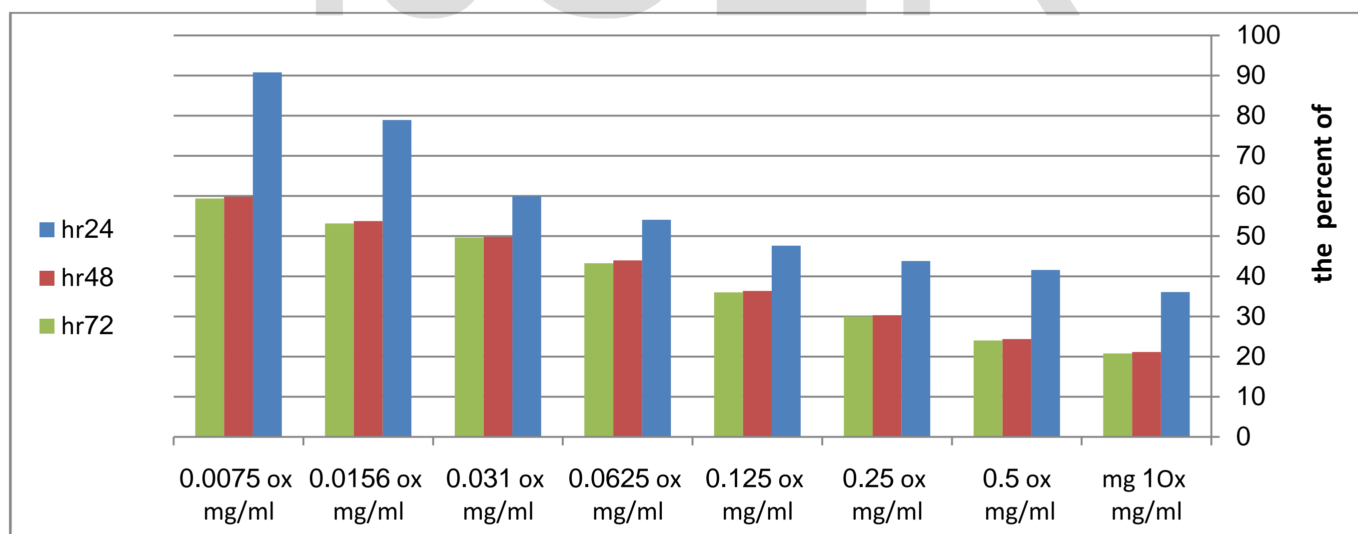

Figure (3-1) cytotoxicity of oxaliplatin on SW-480 cell line (OX= oxaliplatin)

There were significant differences ($P<0.05$) in the percent of viability between 24h and 48h groups of treatment throughout these periods of incubation used in the study as compared to

control group (without oxaliplatin) the viability of SW- 480 cell line decreased through increasing the concentration of oxaliplatin or increasing the time of incubation or both . While there were no- significant differences ($P<0.05$) in the percent of viability between 48hr and 72hr groups of treatment because the oxaliplatin under goes decomposition after reconstitution after 72hr.in vitro.

We can calculate the ID50 of oxaliplatin for 24h (0.125 mg/ml), 48hr (0.031mg/ml) and 72hr of incubation. So the maximum effect of oxaliplatin is after 48hr in vitro

ID50 is the concentration of oxaliplatin that needs to produce 50% viability after specific time of exposure

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3.1.2. Effects of Crocin on SW-480 cell line:

Data in table (3-3) revealed that the viability percent of SW-480 cell line in the presence of different concentrations of Crocin (6, 3, 1, 0.5, 0.25, 0.125, 0.0625 mg/ml) was ranged between (96.5±1.42 - 100.3±4.80) after 24h incubation, (86.35±5.93 - 99.76±3.36) after 48h incubation.

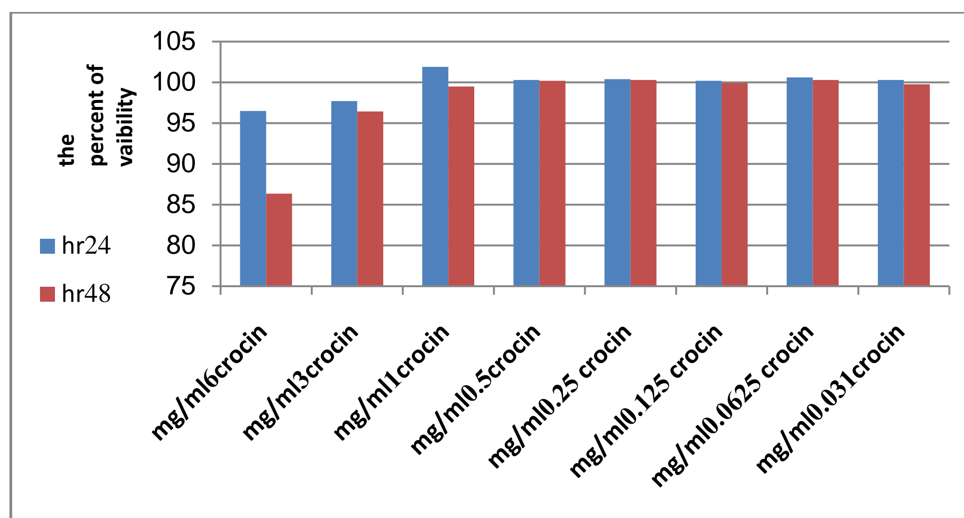

Figure (3-2) cytotoxicity of Crocin for cancer cell in different concentrations and time

There was non-significant differences (P<0.05) in the percent of viability for all groups of treatment throughout the three periods of incubation used in the study as compared to control group (without Crocin).

. Effect of concomitant use of oxaliplatin with different concentration of Crocin on SW-480:

The viability percent of SW-480 cell line for different concentrations of oxaliplatin (1, 0.5, 0.25, 0.125, 0.0625, 0.0312, 0.0156 mg/ml) used in concomitant with different concentration of Crocin (3, 1, 0.5, 0.25, 0.125, 0.0625, 0.0312 mg/ml) for 24h and 48h show in the table (3-4)

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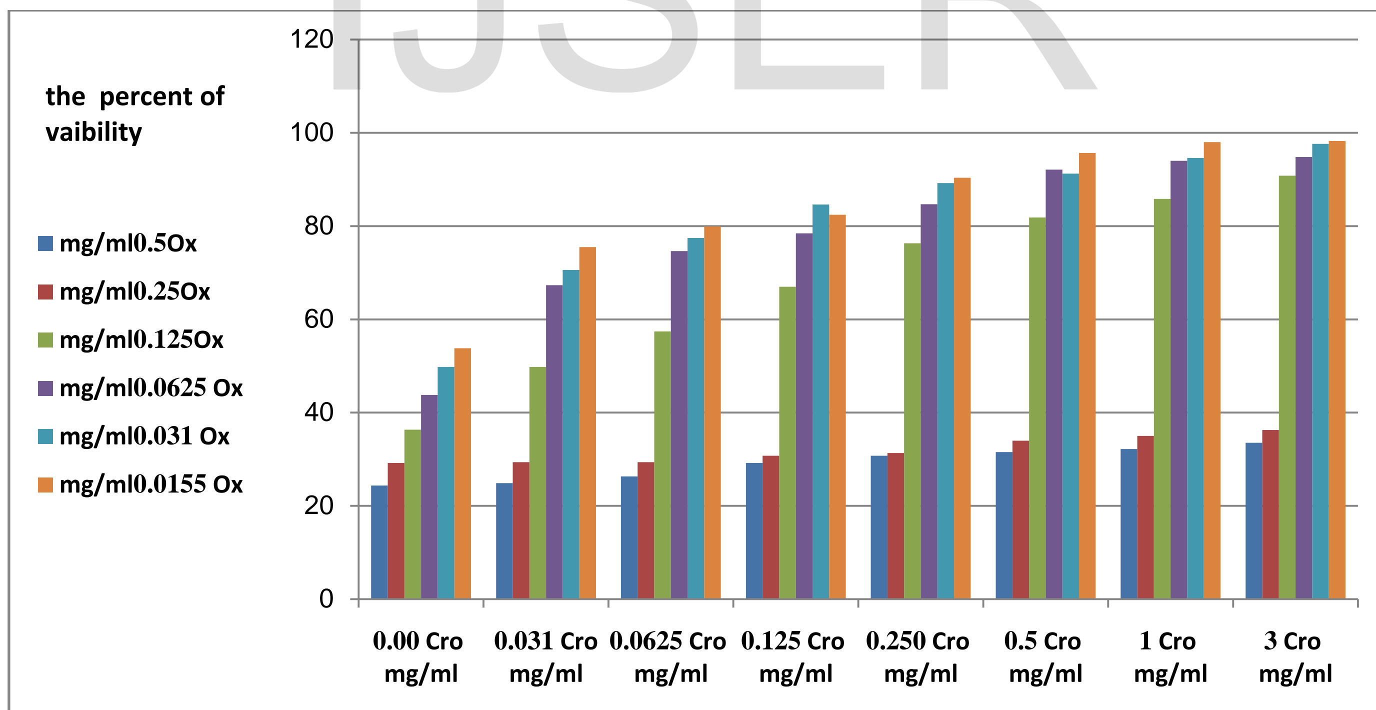

Figure (3-3) viability percent after 48 hour (mix oxaliplatin + crocin) in different concentrations

Discussion:

Cytotoxicity test c.v.:

We can notice that the oxaloplatin has cytotoxicity effect on SW-480 cells in response to the concentration of drug and the time of exposure. So when the concentration of drug increased the viability of the cells decreased or the killing effect of the drug increased (Suzuki et al., 2008).

After 24hr. we can see that the IC50 of the oxaliplatin is (0.125mg/ml), while the IC50 of the oxaliplatin after 48hr. is (0.031mg/ml) and the IC50 after 72hr. is (0.031mg/ml)

So there is a significant reduce in the viability of the SW-480 cells in 24hr. vs. 48hr. and there is a non significant difference in the viability of the SW480 cells between the 48hr. and 72hr. the reasons of this that the oxaliplatin after the reconstitution remained stable for 48hr in vitro and after this period the drug is decomposed . And for these reasons it does not give more effect (Ibrahim and Mauvernay, 1998) (Hospira Company).

So in vitro the maximum effect of this drug, it is reached after 48hr. and for these reasons all our cytotoxicity effect will be measured for 48hr. So the maximum effect of oxaliplatin is after 48hr. and the IC50 is (0.031mg/ml)

For the Crocin we can notice that this crude herbal material does not have any cytotoxic effect on SW480 cell line. Because after the exposure of SW-480 cells to the Crocin in different concentration (6, 3, 1, 0.5, 0.25, 0.125, 0.0625, 0.031 mg/ml) for 24hr. and 48hr.

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the Crocin does not exhibit any cytotoxic effect on the SW-480 cells and this conclusion is fitted with the following researches.

Argyraki et al they said that the Crocin have cytogenic and/ or cytoprotective effect at tested dose (Argyraki et al.).

Lari et al said that the Crocin reduces the hepatotoxicity and protective effect agonist diazion throughout the reduction in inflammation mediator and decrease the caspases activity (Lari et al., 2015)

While there are other researches aren't fitted with these data examples of these researches are as following:

Kim et al said the Crocin and crocetin which is the carotiniod of saffron induce cytotoxic effect (Kim et al., 2014)

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Aung et al also proved that Crocin from Crocus sativus possesses significant anti-proliferation effects on human colorectal cancer cells through the p-53 protein (Aung et al., 2007)

The above researchers said that there is a anti tumor and/ or anti proliferative effect for the Crocin. And these different in the result as we expected is due to either once or more from the following causes;

1. The data or this effect (cytotoxic) effect is measured on the other cell line (ex. HeLa cell line and HepG2). (Tavakkol-Afshari et al., 2008) Study of cytotoxic and apoptogenic properties of saffron extract in human cancer cell lines HeLa HepG2 via the p53 activity.

2. As mentioned in some of these researches they dissolve the Crocin powder in DDW and then applied on the wells that contained the cells as done by (Li et al., 2012) they dissolve the Crocin in DDW while we think is better to dissolve the Crocin in SFM

And this may affect the constituent of the growth media. This may lead to change the concentration of nutrients, minerals, growth factors and co factors and / or PH or the buffer system of the growth media, this disturbance in the media may lead to killing effect to the cells and as we know that a tissue culture experiment is highly sensitive experiment

3. There is another type of crud herbal material that obtained from saffron (ex. Safranal, crocetin) this crud material is examined to have cytotoxic effect on the different cell lines and there is a confusion between the crocetin (have cytotoxic effect) and Crocin (don't have cytotoxic effect) as said by (Li et al., 2012) some time mention Crocin and other mention crocetin.

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In the concomitant use of oxaliplatin and Crocin or mixed the oxaliplatin and Crocin in different concentration for 48hr (maximum effect of oxaliplatin), we can notice that the cytotoxic effect of oxaliplatin decreased when mixed with Crocin for example

The IC50 for oxaliplatin when applied alone is (0.031mg/ml) in 48 hr. while this concentration of oxaliplatin when mixed with (o.5 mg/ml) Crocin the viability is (91.24**±7.9**) in 48hr. and for oxaliplatin when mixed with (0.25 mg/ml) Crocin is **(89.22±6.9)** in 48hr. and for oxaliplatin when mixed with (0.125 mg/ml) Crocin is **(84.61±7.4)** in 48 hr.

The data that obtained we can concluded that the Crocin don't have cytotoxic effect and in reverse, it has cytoprotective effect within this range of concentration

Conclusion:

From our study we can conclude the following:.

1. The Crocin has cytoprotective
2. The Crocin has no anti tumor and anti proliferative activity within these tasted concentration and this cell line

References

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ARGYRAKI, M., ANESTAKIS, D., ZERVAS, C., SINAKOS, Z. & IAKOVIDOU-KRITSI, Z. Cytogenetic behaviour of crocin on cultured lymphocytes from leukemic patients.

AUNG, H., WANG, C., NI, M., FISHBEIN, A., MEHENDALE, S., XIE, J., SHOYAMA, A. & YUAN, C. 2007. Crocin from Crocus sativus possesses significant anti-proliferation effects on human colorectal cancer cells. *Experimental oncology,* 29, 175.

AUNG, H., WANG, C., NI, M., FISHBEIN, A., MEHENDALE, S., XIE, J., SHOYAMA, A. & YUAN, C. 2007. Crocin from Crocus sativus possesses significant anti-proliferation effects on human colorectal cancer cells. *Experimental oncology,* 29, 175.

BATHAIE, S. Z., BOLHASANI, A., HOSHYAR, R., RANJBAR, B., SABOUNI, F. & MOOSAVI-MOVAHEDI, A.-A. 2007. Interaction of saffron carotenoids as anticancer compounds with ctDNA, Oligo (dG. dC) 15, and Oligo (dA. dT) 15. *DNA and cell biology,* 26, 533-540.

FRESHNEY, R. I. & FRESHNEY, M. G. 2004. *Culture of epithelial cells*, John Wiley & Sons.

HADIZADEH, F., MOHAJERI, S. & SEIFI, M. 2010. Extraction and purification of crocin from saffron stigmas employing a simple and efficient crystallization method. *Pakistan Journal of Biological Sciences,* 13, 691.

HOSSEINZADEH, H. & NASSIRI-ASL, M. 2013. Avicenna's (Ibn Sina) the canon of medicine and saffron (Crocus sativus): a review. *Phytotherapy Research,* 27, 475-483.

IBRAHIM, H. & MAUVERNAY, R.-Y. 1998. Pharmaceutically stable preparation of oxaliplatinum. Google Patents.

KIM, S. H., LEE, J. M., KIM, S. C., PARK, C. B. & LEE, P. C. 2014. Proposed cytotoxic mechanisms of the saffron carotenoids crocin and crocetin on cancer cell lines. *Biochemistry and Cell Biology,* 92, 105-111.

LARI, P., ABNOUS, K., IMENSHAHIDI, M., RASHEDINIA, M., RAZAVI, M. & HOSSEINZADEH, H. 2015. Evaluation of diazinon-induced hepatotoxicity and protective effects of crocin. *Toxicology and industrial health,* 31, 367-376.

LI, C.-Y., HUANG, W.-F., WANG, Q.-L., WANG, F., CAI, E., HU, B., DU, J.-C., WANG, J., CHEN, R. & CAI, X.-J. 2012. Crocetin induces cytotoxicity in colon cancer cells via p53-independent mechanisms. *Asian Pacific Journal of Cancer Prevention,* 13, 3757-3761.

MASHMOUL, M., AZLAN, A., KHAZA'AI, H., YUSOF, B. N. M. & NOOR, S. M. 2013. Saffron: A natural potent antioxidant as a promising anti-obesity drug. *Antioxidants,* 2, 293-308.

NAIR, S. C., KURUMBOOR, S. & HASEGAWA, J. 1995. Saffron chemoprevention in biology and medicine: a review. *Cancer Biotherapy & Radiopharmaceuticals,* 10, 257-264.

NOUREINI, S. K. & WINK, M. 2012. Antiproliferative effects of crocin in HepG2 cells by telomerase inhibition and hTERT down-regulation. *Asian Pacific Journal of Cancer Prevention,* 13, 2305-2309.

TAVAKKOL-AFSHARI, J., BROOK, A. & MOUSAVI, S. H. 2008. Study of cytotoxic and apoptogenic properties of saffron extract in human cancer cell lines. *Food and Chemical Toxicology,* 46, 3443-3447.

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XI, L., QIAN, Z., DU, P. & FU, J. 2007. Pharmacokinetic properties of crocin (crocetin digentiobiose ester) following oral administration in rats. *Phytomedicine,* 14, 633-636.

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